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<b>(54) Title:</b> HUMAN MEMBRANE ANTIGEN TM4 SUPERFAMILY PROTEIN AND DNA ENCODING THIS PROTEIN		
<b>(57) Abstract</b>  A human membrane antigen TM4 superfamily protein existing on the osteosarcoma cell surface and a cDNA encoding this protein is provided, said protein being useful as a pharmaceutical for treatment and diagnosis of cancers and also as an antigen for preparation of an antibody against said protein.		

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## DESCRIPTION

## HUMAN MEMBRANE ANTIGEN TM4

SUPERFAMILY PROTEIN AND DNA ENCODING THIS PROTEIN

## TECHNICAL FIELD

The present invention relates to a human membrane antigen TM4 superfamily protein and a cDNA encoding this protein. The protein of the present invention can be used as pharmaceuticals for the treatment and diagnosis of cancers or as an antigen for preparing an antibody against said protein. The cDNA of the present invention can be used as a probe for the gene diagnosis and a gene source for the gene therapy. Furthermore, the cDNA can be used as a gene source for large-scale production of the protein encoded by said cDNA.

## PRIOR ART

## BACKGROUND ART

The type II membrane proteins having transmembrane domains at four sites have been called as the TM4 superfamily and there have been already reported such genes as the CD9 antigen [Boucheix, C. et al., J. Biol. Chem. 266: 117-122 (1991)], the CD37 antigen [Classon, B. J. et al., J. Exp. Med. 169: 1497-1502 (1989)], the CD53 antigen [Amiot, M., J. Immunol. 145: 4322-4325 (1990)], the CD63 antigen [Metzelaar, M. J. et al., J. Biol. Chem., 266: 3239-3245 (1991)], the CD81 antigen [Oren, R. et al., Mol. Cell. Biol. 10: 4007-4015 (1990)], the CD82 antigen [Imai, T., J. Immunol. 149: 2879-2886 (1992)], and so on. All of them have been found out as

the membrane antigens existing on the hemopoietic cell surface and have been used as the markers recognizing the cell population. In addition, such antigens as the CO-029 antigen [Azala, S. et al., Proc. Natl. Acad. Sci. USA 87: 6833-6837 (1990)] and the CD63 antigen have been found out as cancer cell membrane antigens. These antigens have been considered to play an important role in the process for the cell information transmission, for the administration of an antibody against these antigens to the cells induces activation of the cell functions or, conversely, repression of the proliferation. Their detailed functions have not been known, although the possibility as transporters has been pointed out from a similarity of their structure to that of the lacY permease of Escherichia coli and from the possession of transmembrane domains.

Since these membrane antigens are expressed in a manner specific to certain specified cells and cancer cells, antibodies prepared against these antigens can be utilized for a variety of diagnosis or as carriers for the drug delivery system. Also, the cells expressing the membrane antigens by transducing these membrane antigen genes can be applied, for example, to the detection of the corresponding ligands. Hereupon, the existence of many TM4 superfamily proteins other than those mentioned above has been predicted, thereby isolation of a novel gene has been desired for the above-mentioned purposes.

#### DISCLOSURE OF INVENTION

The object of the present invention is to provide

a human membrane antigen TM4 superfamily protein and a cDNA encoding said protein.

As the result of intensive studies, the present inventors were successful in cloning of a human cDNA encoding the human membrane antigen TM4 superfamily protein, thereby completing the present invention. That is to say, the present invention provides a protein containing the amino acid sequence represented by Sequence No. 1 that is a human membrane antigen TM4 superfamily protein. The present invention, also, provides a DNA encoding said protein exemplified as a cDNA containing the base sequence represented by Sequence No. 1.

The protein of the present invention can be obtained, for example, by a method for isolation from human organs, cell lines, etc, a method for preparation of the peptide by the chemical synthesis on the basis of the amino acid sequence of the present invention, or a method for production with the recombinant DNA technology using the DNA encoding the human membrane antigen TM4 superfamily protein of the present invention, wherein the method for obtainment by the recombinant DNA technology is employed preferably. For example, an in vitro expression can be achieved by preparation of an RNA by the in vitro transcription from a vector having the cDNA of the present invention, followed by the in vitro translation using this RNA as a template. Also, the recombination of the translation domain to a suitable expression vector by the method known in the art leads to the expression of a large amount of the encoded protein by using Escherichia coli, Bacillus subtilis, yeasts, animal cells,

and so on.

The protein of the present invention includes peptide fragments (more than 5 amino acid residues) containing any partial amino acid sequence of the amino acid sequence represented by Sequence No. 1. These fragments can be used as antigens for preparation of the antibodies. Particularly, a sequence contained in a region between arginine 148 and glutamic acid 159 that is putatively on the cell membrane surface is suitable for using as an antigen peptide.

The DNA of the present invention includes all DNA encoding said protein. Said DNA can be obtained using the method by chemical synthesis, the method by cDNA cloning, and so on.

The cDNA of the present invention can be cloned from, for example, a cDNA library of the human cell origin. The cDNA is synthesized using as a template a poly(A)<sup>+</sup> RNA extracted from human cells. The human cells may be cells delivered from the human body, for example, by the operation or may be the culture cells. A poly(A)<sup>+</sup> RNA isolated from the human osteosarcoma cell line Saos-2 is used in Examples. The cDNA can be synthesized by using any method selected from the Okayama-Berg method [Okayama, H. and Berg, P., Mol. Cell. Biol. 2: 161-170 (1982)], the Gubler-Hoffman method [Gubler, U. and Hoffman, J. Gene 25: 263-269 (1983)], and so on, but it is preferred to use the capping method [Kato, S. et al., Gene 150: 243-250 (1994)] as illustrated in Examples in order to obtain a full-length clone in an effective manner.

The cloning of the cDNA is performed by the

sequencing of a partial base sequence of the cDNA clone selected at random from the cDNA library and the search of the protein data base by the amino acid sequence predicted from the base sequence. The identification of the cDNA is carried out by determination of the whole base sequence by the sequencing and the protein expression by the in vitro translation.

The cDNA of the present invention is characterized by containing the base sequence represented by Sequence No. 1, as exemplified by that represented by Sequence No. 2 possessing a 1.7-kbp base sequence with a 762-bp open reading frame. This open reading frame codes for a protein consisting of 253 amino acid residues and possessed transmembrane domains at four sites.

Hereupon, the same clone as the cDNA of the present invention can be easily obtained by screening of the human cDNA library constructed from the cell line employed in the present invention, by the use of an oligonucleotide probe synthesized on the basis of the cDNA base sequence depicted in Sequence No. 1 or Sequence No. 2.

In general, the polymorphism due to the individual difference is frequently observed in human genes. Therefore, any cDNA that is subjected to insertion or deletion of one or plural nucleotides and/or substitution with other nucleotides in Sequence No. 1 or Sequence No. 2 shall come within the scope of the present invention.

In a similar manner, any protein that is produced by these modifications comprising insertion or deletion of one or plural nucleotides and/or substitution with other

nucleotides shall come within the scope of the present invention, as far as said protein possesses the activity of the protein having the amino acid sequence represented by Sequence No. 1.

The cDNA of the present invention includes cDNA fragments (more than 10 bp) containing any partial base sequence of the base sequence represented by Sequence No. 1 or No. 2. Also, DNA fragments consisting of a sense chain and an anti-sense chain shall come within this scope. These DNA fragments can be used as the probes for the gene diagnosis.

In addition to the activities and uses described above, the polynucleotides and proteins of the present invention may exhibit one or more of the uses or biological activities (including those associated with assays cited herein) identified below. Uses or activities described for proteins of the present invention may be provided by administration or use of such proteins or by administration or use of polynucleotides encoding such proteins (such as, for example, in gene therapies or vectors suitable for introduction of DNA).

#### Research Uses and Utilities

The polynucleotides provided by the present invention can be used by the research community for various purposes. The polynucleotides can be used to express recombinant protein for analysis, characterization or therapeutic use; as markers for tissues in which the corresponding protein is preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in disease states); as



molecular weight markers on Southern gels; as chromosome markers or tags (when labeled) to identify chromosomes or to map related gene positions; to compare with endogenous DNA sequences in patients to identify potential genetic disorders; as probes to hybridize and thus discover novel, related DNA sequences; as a source of information to derive PCR primers for genetic fingerprinting; as a probe to "subtract-out" known sequences in the process of discovering other novel polynucleotides; for selecting and making oligomers for attachment to a "gene chip" or other support, including for examination of expression patterns; to raise anti-protein antibodies using DNA immunization techniques; and as an antigen to raise anti-DNA antibodies or elicit another immune response. Where the polynucleotide encodes a protein which binds or potentially binds to another protein (such as, for example, in a receptor-ligand interaction), the polynucleotide can also be used in interaction trap assays (such as, for example, that described in Gyuris et al., Cell 75:791-803 (1993)) to identify polynucleotides encoding the other protein with which binding occurs or to identify inhibitors of the binding interaction.

The proteins provided by the present invention can similarly be used in assay to determine biological activity, including in a panel of multiple proteins for high-throughput screening; to raise antibodies or to elicit another immune response; as a reagent (including the labeled reagent) in assays designed to quantitatively determine levels of the protein (or its receptor) in biological fluids; as markers for tissues in which the corresponding protein is

preferentially expressed (either constitutively or at a particular stage of tissue differentiation or development or in a disease state); and, of course, to isolate correlative receptors or ligands. Where the protein binds or potentially binds to another protein (such as, for example, in a receptor-ligand interaction), the protein can be used to identify the other protein with which binding occurs or to identify inhibitors of the binding interaction. Proteins involved in these binding interactions can also be used to screen for peptide or small molecule inhibitors or agonists of the binding interaction.

Any or all of these research utilities are capable of being developed into reagent grade or kit format for commercialization as research products.

Methods for performing the uses listed above are well known to those skilled in the art. References disclosing such methods include without limitation "Molecular Cloning: A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory Press, Sambrook, J., E.F. Fritsch and T. Maniatis eds., 1989, and "Methods in Enzymology: Guide to Molecular Cloning Techniques", Academic Press, Berger, S.L. and A.R. Kimmel eds., 1987.

#### Nutritional Uses

Polynucleotides and proteins of the present invention can also be used as nutritional sources or supplements. Such uses include without limitation use as a protein or amino acid supplement, use as a carbon source, use as a nitrogen source and use as a source of carbohydrate. In such cases the protein or polynucleotide of the invention can

be added to the feed of a particular organism or can be administered as a separate solid or liquid preparation, such as in the form of powder, pills, solutions, suspensions or capsules. In the case of microorganisms, the protein or polynucleotide of the invention can be added to the medium in or on which the microorganism is cultured.

#### Cytokine and Cell Proliferation/Differentiation

##### Activity

A protein of the present invention may exhibit cytokine, cell proliferation (either inducing or inhibiting) or cell differentiation (either inducing or inhibiting) activity or may induce production of other cytokines in certain cell populations. Many protein factors discovered to date, including all known cytokines, have exhibited activity in one or more factor dependent cell proliferation assays, and hence the assays serve as a convenient confirmation of cytokine activity. The activity of a protein of the present invention is evidenced by any one of a number of routine factor dependent cell proliferation assays for cell lines including, without limitation, 32D, DA2, DA1G, T10, B9, B9/11, BaF3, MC9/G, M+ (preB M+), 2E8, RB5, DA1, 123, T1165, HT2, CTLL2, TF-1, Mo7e and CMK.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Assays for T-cell or thymocyte proliferation include without limitation those described in: Current Protocols in Immunology, Ed by J. E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3. In

Vitro assays for Mouse Lymphocyte Function 3.1-3.19; Chapter 7, Immunologic studies in Humans); Takai et al., J. Immunol. 137:3494-3500, 1986; Bertagnolli et al., J. Immunol. 145:1706-1712, 1990; Bertagnolli et al., Cellular Immunology 133:327-341, 1991; Bertagnolli, et al., J. Immunol. 149:3778-3783, 1992; Bowman et al., J. Immunol. 152: 1756-1761, 1994.

Assays for cytokine production and/or proliferation of spleen cells, lymph node cells or thymocytes include, without limitation, those described in: Polyclonal T cell stimulation, Kruisbeek, A.M. and Shevach, E.M. In Current Protocols in Immunology. J.E.e.a. Coligan eds. Vol 1 pp. 3.12.1-3.12.14, John Wiley and Sons, Toronto. 1994; and Measurement of mouse and human Interferon  $\gamma$ , Schreiber, R.D. In Current Protocols in Immunology. J.E.e.a. Coligan eds. Vol 1 pp. 6.8.1-6.8.8, John Wiley and Sons, Toronto. 1994.

Assays for proliferation and differentiation of hematopoietic and lymphopoietic cells include, without limitation, those described in: Measurement of Human and Murine Interleukin 2 and Interleukin 4, Bottomly, K., Davis, L.S. and Lipsky, P.E. In Current Protocols in Immunology. J.E.e.a. Coligan eds. Vol 1 pp. 6.3.1-6.3.12, John Wiley and Sons, Toronto. 1991; deVries et al., J. Exp. Med. 173:1205-1211, 1991; Moreau et al., Nature 336:690-692, 1988; Greenberger et al., Proc. Natl. Acad. Sci. U.S.A. 80:2931-2938, 1983; Measurement of mouse and human interleukin 6 -Nordan, R. In Current Protocols in Immunology. J.E.e.a. Coligan eds. Vol 1 pp. 6.6.1-6.6.5, John Wiley and Sons Toronto. 1991; Smith et al., Proc. Natl. Acad. Sci.

U.S.A. 83:1857-1861, 1986; Measurement of human Interleukin 11 - Bennett, F., Giannotti, J., Clark, S.C. and Turner, K. J. In Current Protocols in Immunology. J.E.e.a. Coligan eds. Vol 1 pp. 6.15.1 John Wiley and Sons, Toronto. 1991; Measurement of mouse and human Interleukin 9 - Ciarletta, A., Giannotti, J., Clark, S.C. and Turner, K.J. In Current Protocols in Immunology. J.E.e.a. Coligan eds. Vol 1 pp. 6.13.1, John Wiley and Sons, Toronto. 1991.

Assays for T-cell clone responses to antigens (which will identify, among others, proteins that affect APC-T cell interactions as well as direct T-cell effects by measuring proliferation and cytokine production) include, without limitation, those described in: Current Protocols in Immunology, Ed by J. E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, In Vitro assays for Mouse Lymphocyte Function; Chapter 6, Cytokines and their cellular receptors; Chapter 7, Immunologic studies in Humans); Weinberger et al., Proc. Natl. Acad. Sci. USA 77:6091-6095, 1980; Weinberger et al., Eur. J. Immun. 11:405-411, 1981; Takai et al., J. Immunol. 137:3494-3500, 1986; Takai et al., J. Immunol. 140:508-512, 1988.

#### Immune Stimulating or Suppressing Activity

A protein of the present invention may also exhibit immune stimulating or immune suppressing activity, including without limitation the activities for which assays are described herein. A protein may be useful in the treatment of various immune deficiencies and disorders (including severe combined immunodeficiency (SCID)), e.g., in regulating

(up or down) growth and proliferation of T and/or B lymphocytes, as well as effecting the cytolytic activity of NK cells and other cell populations. These immune deficiencies may be genetic or be caused by viral (e.g., HIV) as well as bacterial or fungal infections, or may result from autoimmune disorders. More specifically, infectious diseases caused by viral, bacterial, fungal or other infection may be treatable using a protein of the present invention, including infections by HIV, hepatitis viruses, herpesviruses, mycobacteria, Leishmania spp., malaria spp. and various fungal infections such as candidiasis. Of course, in this regard, a protein of the present invention may also be useful where a boost to the immune system generally may be desirable, i.e., in the treatment of cancer.

Autoimmune disorders which may be treated using a protein of the present invention include, for example, connective tissue disease, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, autoimmune pulmonary inflammation, Guillain-Barre syndrome, autoimmune thyroiditis, insulin dependent diabetes mellitus, myasthenia gravis, graft-versus-host disease and autoimmune inflammatory eye disease. Such a protein of the present invention may also be useful in the treatment of allergic reactions and conditions, such as asthma (particularly allergic asthma) or other respiratory problems. Other conditions, in which immune suppression is desired (including, for example, organ transplantation), may also be treatable using a protein of the present invention.

Using the proteins of the invention it may also be

possible to immune responses, in a number of ways. Down regulation may be in the form of inhibiting or blocking an immune response already in progress or may involve preventing the induction of an immune response. The functions of activated T cells may be inhibited by suppressing T cell responses or by inducing specific tolerance in T cells, or both. Immunosuppression of T cell responses is generally an active, non-antigen-specific, process which requires continuous exposure of the T cells to the suppressive agent. Tolerance, which involves inducing non-responsiveness or anergy in T cells, is distinguishable from immunosuppression in that it is generally antigen-specific and persists after exposure to the tolerizing agent has ceased. Operationally, tolerance can be demonstrated by the lack of a T cell response upon reexposure to specific antigen in the absence of the tolerizing agent.

Down regulating or preventing one or more antigen functions (including without limitation B lymphocyte antigen functions (such as , for example, B7)), e.g., preventing high level lymphokine synthesis by activated T cells, will be useful in situations of tissue, skin and organ transplantation and in graft-versus-host disease (GVHD). For example, blockage of T cell function should result in reduced tissue destruction in tissue transplantation. Typically, in tissue transplants, rejection of the transplant is initiated through its recognition as foreign by T cells, followed by an immune reaction that destroys the transplant. The administration of a molecule which inhibits or blocks interaction of a B7 lymphocyte antigen with its natural

ligand(s) on immune cells (such as a soluble, monomeric form of a peptide having B7-2 activity alone or in conjunction with a monomeric form of a peptide having an activity of another B lymphocyte antigen (e.g., B7-1, B7-3) or blocking antibody), prior to transplantation can lead to the binding of the molecule to the natural ligand(s) on the immune cells without transmitting the corresponding costimulatory signal. Blocking B lymphocyte antigen function in this matter prevents cytokine synthesis by immune cells, such as T cells, and thus acts as an immunosuppressant. Moreover, the lack of costimulation may also be sufficient to anergize the T cells, thereby inducing tolerance in a subject. Induction of long-term tolerance by B lymphocyte antigen-blocking reagents may avoid the necessity of repeated administration of these blocking reagents. To achieve sufficient immunosuppression or tolerance in a subject, it may also be necessary to block the function of a combination of B lymphocyte antigens.

The efficacy of particular blocking reagents in preventing organ transplant rejection or GVHD can be assessed using animal models that are predictive of efficacy in humans. Examples of appropriate systems which can be used include allogeneic cardiac grafts in rats and xenogeneic pancreatic islet cell grafts in mice, both of which have been used to examine the immunosuppressive effects of CTLA4Ig fusion proteins in vivo as described in Lenschow et al., *Science* 257:789-792 (1992) and Turka et al., *Proc. Natl. Acad. Sci USA*, 89:11102-11105 (1992). In addition, murine models of GVHD (see Paul ed., *Fundamental Immunology*, Raven Press, New York, 1989, pp. 846-847) can be used to determine



the effect of blocking B lymphocyte antigen function in vivo on the development of that disease.

Blocking antigen function may also be therapeutically useful for treating autoimmune diseases. Many autoimmune disorders are the result of inappropriate activation of T cells that are reactive against self tissue and which promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Preventing the activation of autoreactive T cells may reduce or eliminate disease symptoms. Administration of reagents which block costimulation of T cells by disrupting receptor:ligand interactions of B lymphocyte antigens can be used to inhibit T cell activation and prevent production of autoantibodies or T cell-derived cytokines which may be involved in the disease process. Additionally, blocking reagents may induce antigen-specific tolerance of autoreactive T cells which could lead to long-term relief from the disease. The efficacy of blocking reagents in preventing or alleviating autoimmune disorders can be determined using a number of well-characterized animal models of human autoimmune diseases. Examples include murine experimental autoimmune encephalitis, systemic lupus erythmatosis in MRL/lpr/lpr mice or NZB hybrid mice, murine autoimmune collagen arthritis, diabetes mellitus in NOD mice and BB rats, and murine experimental myasthenia gravis (see Paul ed., Fundamental Immunology, Raven Press, New York, 1989, pp. 840-856).

Upregulation of an antigen function (preferably a B lymphocyte antigen function), as a means of up regulating

immune responses, may also be useful in therapy. Upregulation of immune responses may be in the form of enhancing an existing immune response or eliciting an initial immune response. For example, enhancing an immune response through stimulating B lymphocyte antigen function may be useful in cases of viral infection. In addition, systemic viral diseases such as influenza, the common cold, and encephalitis might be alleviated by the administration of stimulatory forms of B lymphocyte antigens systemically.

Alternatively, anti-viral immune responses may be enhanced in an infected patient by removing T cells from the patient, costimulating the T cells in vitro with viral antigen-pulsed APCs either expressing a peptide of the present invention or together with a stimulatory form of a soluble peptide of the present invention and reintroducing the in vitro activated T cells into the patient. Another method of enhancing anti-viral immune responses would be to isolate infected cells from a patient, transfect them with a nucleic acid encoding a protein of the present invention as described herein such that the cells express all or a portion of the protein on their surface, and reintroduce the transfected cells into the patient. The infected cells would now be capable of delivering a costimulatory signal to, and thereby activate, T cells in vivo.

In another application, up regulation or enhancement of antigen function (preferably B lymphocyte antigen function) may be useful in the induction of tumor immunity. Tumor cells (e.g. sarcoma, melanoma, lymphoma,

leukemia, neuroblastoma, carcinoma) transfected with a nucleic acid encoding at least one peptide of the present invention can be administered to a subject to overcome tumor-specific tolerance in the subject. If desired, the tumor cell can be transfected to express a combination of peptides. For example, tumor cells obtained from a patient can be transfected *ex vivo* with an expression vector directing the expression of a peptide having B7-2-like activity alone, or in conjunction with a peptide having B7-1-like activity and/or B7-3-like activity. The transfected tumor cells are returned to the patient to result in expression of the peptides on the surface of the transfected cell. Alternatively, gene therapy techniques can be used to target a tumor cell for transfection *in vivo*.

The presence of the peptide of the present invention having the activity of a B lymphocyte antigen(s) on the surface of the tumor cell provides the necessary costimulation signal to T cells to induce a T cell mediated immune response against the transfected tumor cells. In addition, tumor cells which lack MHC class I or MHC class II molecules, or which fail to reexpress sufficient amounts of MHC class I or MHC class II molecules, can be transfected with nucleic acid encoding all or a portion of (e.g., a cytoplasmic-domain truncated portion) of an MHC class I  $\alpha$  chain protein and  $\beta_2$  microglobulin protein or an MHC class II  $\alpha$  chain protein and an MHC class II  $\beta$  chain protein to thereby express MHC class I or MHC class II proteins on the cell surface. Expression of the appropriate class I or class II MHC in conjunction with a peptide having the activity of

a B lymphocyte antigen (e.g., B7-1, B7-2, B7-3) induces a T cell mediated immune response against the transfected tumor cell. Optionally, a gene encoding an antisense construct which blocks expression of an MHC class II associated protein, such as the invariant chain, can also be cotransfected with a DNA encoding a peptide having the activity of a B lymphocyte antigen to promote presentation of tumor associated antigens and induce tumor specific immunity. Thus, the induction of a T cell mediated immune response in a human subject may be sufficient to overcome tumor-specific tolerance in the subject.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Suitable assays for thymocyte or splenocyte cytotoxicity include, without limitation, those described in: Current Protocols in Immunology, Ed by J. E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, In Vitro assays for Mouse Lymphocyte Function 3.1-3.19; Chapter 7, Immunologic studies in Humans); Herrmann et al., Proc. Natl. Acad. Sci. USA 78:2488-2492, 1981; Herrmann et al., J. Immunol. 128:1968-1974, 1982; Handa et al., J. Immunol. 135:1564-1572, 1985; Takai et al., J. Immunol. 137:3494-3500, 1986; Takai et al., J. Immunol. 140:508-512, 1988; Herrmann et al., Proc. Natl. Acad. Sci. USA 78:2488-2492, 1981; Herrmann et al., J. Immunol. 128:1968-1974, 1982; Handa et al., J. Immunol. 135:1564-1572, 1985; Takai et al., J. Immunol. 137:3494-3500, 1986; Bowman et al., J. Virology 61:1992-1998; Takai et al., J. Immunol.

140:508-512, 1988; Bertagnolli et al., Cellular Immunology 133:327-341, 1991; Brown et al., J. Immunol. 153:3079-3092, 1994.

Assays for T-cell-dependent immunoglobulin responses and isotype switching (which will identify, among others, proteins that modulate T-cell dependent antibody responses and that affect Th1/Th2 profiles) include, without limitation, those described in: Maliszewski, J. Immunol. 144:3028-3033, 1990; and Assays for B cell function: In vitro antibody production, Mond, J.J. and Brunswick, M. In Current Protocols in Immunology. J.E.e.a. Coligan eds. Vol 1 pp. 3.8.1-3.8.16, John Wiley and Sons, Toronto. 1994.

Mixed lymphocyte reaction (MLR) assays (which will identify, among others, proteins that generate predominantly Th1 and CTL responses) include, without limitation, those described in: Current Protocols in Immunology, Ed by J. E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 3, In Vitro assays for Mouse Lymphocyte Function 3.1-3.19; Chapter 7, Immunologic studies in Humans); Takai et al., J. Immunol. 137:3494-3500, 1986; Takai et al., J. Immunol. 140:508-512, 1988; Bertagnolli et al., J. Immunol. 149:3778-3783, 1992.

Dendritic cell-dependent assays (which will identify, among others, proteins expressed by dendritic cells that activate naive T-cells) include, without limitation, those described in: Guery et al., J. Immunol. 134:536-544, 1995; Inaba et al., Journal of Experimental Medicine 173:549-559, 1991; Macatonia et al., Journal of Immunology

154:5071-5079, 1995; Porgador et al., Journal of Experimental Medicine 182:255-260, 1995; Nair et al., Journal of Virology 67:4062-4069, 1993; Huang et al., Science 264:961-965, 1994; Macatonia et al., Journal of Experimental Medicine 169:1255-1264, 1989; Bhardwaj et al., Journal of Clinical Investigation 94:797-807, 1994; and Inaba et al., Journal of Experimental Medicine 172:631-640, 1990.

Assays for lymphocyte survival/apoptosis (which will identify, among others, proteins that prevent apoptosis after superantigen induction and proteins that regulate lymphocyte homeostasis) include, without limitation, those described in: Darzynkiewicz et al., Cytometry 13:795-808, 1992; Gorczyca et al., Leukemia 7:659-670, 1993; Gorczyca et al., Cancer Research 53:1945-1951, 1993; Itoh et al., Cell 66:233-243, 1991; Zacharchuk, Journal of Immunology 145:4037-4045, 1990; Zamai et al., Cytometry 14:891-897, 1993; Gorczyca et al., International Journal of Oncology 1:639-648, 1992.

Assays for proteins that influence early steps of T-cell commitment and development include, without limitation, those described in: Antica et al., Blood 84:111-117, 1994; Fine et al., Cellular Immunology 155:111-122, 1994; Galy et al., Blood 85:2770-2778, 1995; Toki et al., Proc. Nat. Acad. Sci. USA 88:7548-7551, 1991.

#### Hematopoiesis Regulating Activity

A protein of the present invention may be useful in regulation of hematopoiesis and, consequently, in the treatment of myeloid or lymphoid cell deficiencies. Even marginal biological activity in support of colony forming

cells or of factor-dependent cell lines indicates involvement in regulating hematopoiesis, e.g. in supporting the growth and proliferation of erythroid progenitor cells alone or in combination with other cytokines, thereby indicating utility, for example, in treating various anemias or for use in conjunction with irradiation/chemotherapy to stimulate the production of erythroid precursors and/or erythroid cells; in supporting the growth and proliferation of myeloid cells such as granulocytes and monocytes/macrophages (i.e., traditional CSF activity) useful, for example, in conjunction with chemotherapy to prevent or treat consequent myelo-suppression; in supporting the growth and proliferation of megakaryocytes and consequently of platelets thereby allowing prevention or treatment of various platelet disorders such as thrombocytopenia, and generally for use in place of or complimentary to platelet transfusions; and/or in supporting the growth and proliferation of hematopoietic stem cells which are capable of maturing to any and all of the above-mentioned hematopoietic cells and therefore find therapeutic utility in various stem cell disorders (such as those usually treated with transplantation, including, without limitation, aplastic anemia and paroxysmal nocturnal hemoglobinuria), as well as in repopulating the stem cell compartment post irradiation/chemotherapy, either in-vivo or ex-vivo (i.e., in conjunction with bone marrow transplantation or with peripheral progenitor cell transplantation (homologous or heterologous)) as normal cells or genetically manipulated for gene therapy.

The activity of a protein of the invention may,

among other means, be measured by the following methods:

Suitable assays for proliferation and differentiation of various hematopoietic lines are cited above.

Assays for embryonic stem cell differentiation (which will identify, among others, proteins that influence embryonic differentiation hematopoiesis) include, without limitation, those described in: Johansson et al. Cellular Biology 15:141-151, 1995; Keller et al., Molecular and Cellular Biology 13:473-486, 1993; McClanahan et al., Blood 81:2903-2915, 1993.

Assays for stem cell survival and differentiation (which will identify, among others, proteins that regulate lympho-hematopoiesis) include, without limitation, those described in: Methylcellulose colony forming assays, Freshney, M.G. In Culture of Hematopoietic Cells. R.I. Freshney, et al. eds. Vol pp. 265-268, Wiley-Liss, Inc., New York, NY. 1994; Hirayama et al., Proc. Natl. Acad. Sci. USA 89:5907-5911, 1992; Primitive hematopoietic colony forming cells with high proliferative potential, McNiece, I.K. and Briddell, R.A. In Culture of Hematopoietic Cells. R.I. Freshney, et al. eds. Vol pp. 23-39, Wiley-Liss, Inc., New York, NY. 1994; Neben et al., Experimental Hematology 22:353-359, 1994; Cobblestone area forming cell assay, Ploemacher, R.E. In Culture of Hematopoietic Cells. R.I. Freshney, et al. eds. Vol pp. 1-21, Wiley-Liss, Inc., New York, NY. 1994; Long term bone marrow cultures in the presence of stromal cells, Spooncer, E., Dexter, M. and Allen, T. In Culture of Hematopoietic Cells. R.I. Freshney,



et al. eds. Vol pp. 163-179, Wiley-Liss, Inc., New York, NY. 1994; Long term culture initiating cell assay, Sutherland, H.J. In Culture of Hematopoietic Cells. R.I. Freshney, et al. eds. Vol pp. 139-162, Wiley-Liss, Inc., New York, NY. 1994.

#### Tissue Growth Activity

A protein of the present invention also may have utility in compositions used for bone, cartilage, tendon, ligament and/or nerve tissue growth or regeneration, as well as for wound healing and tissue repair and replacement, and in the treatment of burns, incisions and ulcers.

A protein of the present invention, which induces cartilage and/or bone growth in circumstances where bone is not normally formed, has application in the healing of bone fractures and cartilage damage or defects in humans and other animals. Such a preparation employing a protein of the invention may have prophylactic use in closed as well as open fracture reduction and also in the improved fixation of artificial joints. De novo bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma induced, or oncologic resection induced craniofacial defects, and also is useful in cosmetic plastic surgery.

A protein of this invention may also be used in the treatment of periodontal disease, and in other tooth repair processes. Such agents may provide an environment to attract bone-forming cells, stimulate growth of bone-forming cells or induce differentiation of progenitors of bone-forming cells. A protein of the invention may also be useful in the treatment of osteoporosis or osteoarthritis, such as through stimulation of bone and/or cartilage repair or by blocking

inflammation or processes of tissue destruction (collagenase activity, osteoclast activity, etc.) mediated by inflammatory processes.

Another category of tissue regeneration activity that may be attributable to the protein of the present invention is tendon/ligament formation. A protein of the present invention, which induces tendon/ligament-like tissue or other tissue formation in circumstances where such tissue is not normally formed, has application in the healing of tendon or ligament tears, deformities and other tendon or ligament defects in humans and other animals. Such a preparation employing a tendon/ligament-like tissue inducing protein may have prophylactic use in preventing damage to tendon or ligament tissue, as well as use in the improved fixation of tendon or ligament to bone or other tissues, and in repairing defects to tendon or ligament tissue. De novo tendon/ligament-like tissue formation induced by a composition of the present invention contributes to the repair of congenital, trauma induced, or other tendon or ligament defects of other origin, and is also useful in cosmetic plastic surgery for attachment or repair of tendons or ligaments. The compositions of the present invention may provide an environment to attract tendon- or ligament-forming cells, stimulate growth of tendon- or ligament-forming cells, induce differentiation of progenitors of tendon- or ligament-forming cells, or induce growth of tendon/ligament cells or progenitors ex vivo for return in vivo to effect tissue repair. The compositions of the invention may also be useful in the treatment of tendinitis, carpal tunnel syndrome

and other tendon or ligament defects. The compositions may also include an appropriate matrix and/or sequestering agent as a carrier as is well known in the art.

The protein of the present invention may also be useful for proliferation of neural cells and for regeneration of nerve and brain tissue, i.e. for the treatment of central and peripheral nervous system diseases and neuropathies, as well as mechanical and traumatic disorders, which involve degeneration, death or trauma to neural cells or nerve tissue. More specifically, a protein may be used in the treatment of diseases of the peripheral nervous system, such as peripheral nerve injuries, peripheral neuropathy and localized neuropathies, and central nervous system diseases, such as Alzheimer's, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Shy-Drager syndrome. Further conditions which may be treated in accordance with the present invention include mechanical and traumatic disorders, such as spinal cord disorders, head trauma and cerebrovascular diseases such as stroke. Peripheral neuropathies resulting from chemotherapy or other medical therapies may also be treatable using a protein of the invention.

Proteins of the invention may also be useful to promote better or faster closure of non-healing wounds, including without limitation pressure ulcers, ulcers associated with vascular insufficiency, surgical and traumatic wounds, and the like.

It is expected that a protein of the present invention may also exhibit activity for generation or

regeneration of other tissues, such as organs (including, for example, pancreas, liver, intestine, kidney, skin, endothelium), muscle (smooth, skeletal or cardiac) and vascular (including vascular endothelium) tissue, or for promoting the growth of cells comprising such tissues. Part of the desired effects may be by inhibition or modulation of fibrotic scarring to allow normal tissue to regenerate. A protein of the invention may also exhibit angiogenic activity.

A protein of the present invention may also be useful for gut protection or regeneration and treatment of lung or liver fibrosis, reperfusion injury in various tissues, and conditions resulting from systemic cytokine damage.

A protein of the present invention may also be useful for promoting or inhibiting differentiation of tissues described above from precursor tissues or cells; or for inhibiting the growth of tissues described above.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Assays for tissue generation activity include, without limitation, those described in: International Patent Publication No. W095/16035 (bone, cartilage, tendon); International Patent Publication No. W095/05846 (nerve, neuronal); International Patent Publication No. W091/07491 (skin, endothelium ).

Assays for wound healing activity include, without limitation, those described in: Winter, Epidermal Wound Healing pps 71-112 (Maibach, HI and Rovee, DT, eds.), Year

Book Medical Publishers, Inc., Chicago, as modified by Eaglstein and Mertz, J. Invest. Dermatol 71:382-84 (1978).

Activin/Inhibin Activity

A protein of the present invention may also exhibit activin- or inhibin-related activities. Inhibins are characterized by their ability to inhibit the release of follicle stimulating hormone (FSH), while activins are characterized by their ability to stimulate the release of follicle stimulating hormone (FSH). Thus, a protein of the present invention, alone or in heterodimers with a member of the inhibin  $\alpha$  family, may be useful as a contraceptive based on the ability of inhibins to decrease fertility in female mammals and decrease spermatogenesis in male mammals. Administration of sufficient amounts of other inhibins can induce infertility in these mammals. Alternatively, the protein of the invention, as a homodimer or as a heterodimer with other protein subunits of the inhibin- $\beta$  group, may be useful as a fertility inducing therapeutic, based upon the ability of activin molecules in stimulating FSH release from cells of the anterior pituitary. See, for example, United States Patent 4,798,885. A protein of the invention may also be useful for advancement of the onset of fertility in sexually immature mammals, so as to increase the lifetime reproductive performance of domestic animals such as cows, sheep and pigs.

The activity of a protein of the invention may, among other means, be measured by the following methods:

Assays for activin/inhibin activity include, without limitation, those described in: Vale et al.,

Endocrinology 91:562-572, 1972; Ling et al., Nature 321:779-782, 1986; Vale et al., Nature 321:776-779, 1986; Mason et al., Nature 318:659-663, 1985; Forage et al., Proc. Natl. Acad. Sci. USA 83:3091-3095, 1986.

#### Chemotactic/Chemokinetic Activity

A protein of the present invention may have chemotactic or chemokinetic activity (e.g., act as a chemokine) for mammalian cells, including, for example, monocytes, fibroblasts, neutrophils, T-cells, mast cells, eosinophils, epithelial and/or endothelial cells. Chemotactic and chemokinetic proteins can be used to mobilize or attract a desired cell population to a desired site of action. Chemotactic or chemokinetic proteins provide particular advantages in treatment of wounds and other trauma to tissues, as well as in treatment of localized infections. For example, attraction of lymphocytes, monocytes or neutrophils to tumors or sites of infection may result in improved immune responses against the tumor or infecting agent.

A protein or peptide has chemotactic activity for a particular cell population if it can stimulate, directly or indirectly, the directed orientation or movement of such cell population. Preferably, the protein or peptide has the ability to directly stimulate directed movement of cells. Whether a particular protein has chemotactic activity for a population of cells can be readily determined by employing such protein or peptide in any known assay for cell chemotaxis.

The activity of a protein of the invention may,

among other means, be measured by the following methods:

Assays for chemotactic activity (which will identify proteins that induce or prevent chemotaxis) consist of assays that measure the ability of a protein to induce the migration of cells across a membrane as well as the ability of a protein to induce the adhesion of one cell population to another cell population. Suitable assays for movement and adhesion include, without limitation, those described in: Current Protocols in Immunology, Ed by J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W. Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 6.12, Measurement of alpha and beta Chemokines 6.12.1-6.12.28; Taub et al. J. Clin. Invest. 95:1370-1376, 1995; Lind et al. APMIS 103:140-146, 1995; Muller et al Eur. J. Immunol. 25: 1744-1748; Gruber et al. J. of Immunol. 152:5860-5867, 1994; Johnston et al. J. of Immunol. 153: 1762-1768, 1994.

#### Hemostatic and Thrombolytic Activity

A protein of the invention may also exhibit hemostatic or thrombolytic activity. As a result, such a protein is expected to be useful in treatment of various coagulation disorders (including hereditary disorders, such as hemophilias) or to enhance coagulation and other hemostatic events in treating wounds resulting from trauma, surgery or other causes. A protein of the invention may also be useful for dissolving or inhibiting formation of thromboses and for treatment and prevention of conditions resulting therefrom (such as, for example, infarction of cardiac and central nervous system vessels (e.g., stroke)).

The activity of a protein of the invention may, among other means, be measured by the following methods:

Assay for hemostatic and thrombolytic activity include, without limitation, those described in: Linet et al., J. Clin. Pharmacol. 26:131-140, 1986; Burdick et al., Thrombosis Res. 45:413-419, 1987; Humphrey et al., Fibrinolysis 5:71-79 (1991); Schaub, Prostaglandins 35:467-474, 1988.

#### Receptor/Ligand Activity

A protein of the present invention may also demonstrate activity as receptors, receptor ligands or inhibitors or agonists of receptor/ligand interactions. Examples of such receptors and ligands include, without limitation, cytokine receptors and their ligands, receptor kinases and their ligands, receptor phosphatases and their ligands, receptors involved in cell-cell interactions and their ligands (including without limitation, cellular adhesion molecules (such as selectins, integrins and their ligands) and receptor/ligand pairs involved in antigen presentation, antigen recognition and development of cellular and humoral immune responses). Receptors and ligands are also useful for screening of potential peptide or small molecule inhibitors of the relevant receptor/ligand interaction. A protein of the present invention (including, without limitation, fragments of receptors and ligands) may themselves be useful as inhibitors of receptor/ligand interactions.

The activity of a protein of the invention may, among other means, be measured by the following methods:



Suitable assays for receptor-ligand activity include without limitation those described in: Current Protocols in Immunology, Ed by J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W. Strober, Pub. Greene Publishing Associates and Wiley-Interscience (Chapter 7.28, Measurement of Cellular Adhesion under static conditions 7.28.1-7.28.22), Takai et al., Proc. Natl. Acad. Sci. USA 84:6864-6868, 1987; Bierer et al., J. Exp. Med. 168:1145-1156, 1988; Rosenstein et al., J. Exp. Med. 169:149-160 1989; Stoltenberg et al., J. Immunol. Methods 175:59-68, 1994; Stitt et al., Cell 80:661-670, 1995.

#### Anti-Inflammatory Activity

Proteins of the present invention may also exhibit anti-inflammatory activity. The anti-inflammatory activity may be achieved by providing a stimulus to cells involved in the inflammatory response, by inhibiting or promoting cell-cell interactions (such as, for example, cell adhesion), by inhibiting or promoting chemotaxis of cells involved in the inflammatory process, inhibiting or promoting cell extravasation, or by stimulating or suppressing production of other factors which more directly inhibit or promote an inflammatory response. Proteins exhibiting such activities can be used to treat inflammatory conditions including chronic or acute conditions), including without limitation inflammation associated with infection (such as septic shock, sepsis or systemic inflammatory response syndrome (SIRS)), ischemia-reperfusion injury, endotoxin lethality, arthritis, complement-mediated hyperacute rejection, nephritis, cytokine or chemokine-induced lung injury, inflammatory bowel disease.

Crohn's disease or resulting from over production of cytokines such as TNF or IL-1. Proteins of the invention may also be useful to treat anaphylaxis and hypersensitivity to an antigenic substance or material.

#### Tumor Inhibition Activity

In addition to the activities described above for immunological treatment or prevention of tumors, a protein of the invention may exhibit other anti-tumor activities. A protein may inhibit tumor growth directly or indirectly (such as, for example, via ADCC). A protein may exhibit its tumor inhibitory activity by acting on tumor tissue or tumor precursor tissue, by inhibiting formation of tissues necessary to support tumor growth (such as, for example, by inhibiting angiogenesis), by causing production of other factors, agents or cell types which inhibit tumor growth, or by suppressing, eliminating or inhibiting factors, agents or cell types which promote tumor growth

#### Other Activities

A protein of the invention may also exhibit one or more of the following additional activities or effects: inhibiting the growth, infection or function of, or killing, infectious agents, including, without limitation, bacteria, viruses, fungi and other parasites; effecting (suppressing or enhancing) bodily characteristics, including, without limitation, height, weight, hair color, eye color, skin, fat to lean ratio or other tissue pigmentation, or organ or body part size or shape (such as, for example, breast augmentation or diminution, change in bone form or shape); effecting biorhythms or cardiac cycles or rhythms; effecting the

fertility of male or female subjects; effecting the metabolism, catabolism, anabolism, processing, utilization, storage or elimination of dietary fat, lipid, protein, carbohydrate, vitamins, minerals, cofactors or other nutritional factors or component(s); effecting behavioral characteristics, including, without limitation, appetite, libido, stress, cognition (including cognitive disorders), depression (including depressive disorders) and violent behaviors; providing analgesic effects or other pain reducing effects; promoting differentiation and growth of embryonic stem cells in lineages other than hematopoietic lineages; hormonal or endocrine activity; in the case of enzymes, correcting deficiencies of the enzyme and treating deficiency-related diseases; treatment of hyperproliferative disorders (such as, for example, psoriasis); immunoglobulin-like activity (such as, for example, the ability to bind antigens or complement); and the ability to act as an antigen in a vaccine composition to raise an immune response against such protein or another material or entity which is cross-reactive with such protein.

#### BRIEF DESCRIPTION OF DRAWINGS

Figure 1: A figure depicting the structure of the plasmid pHP00966.

Figure 2: A figure depicting the hydrophobicity profile of the protein of the present invention.

#### BEST MODE FOR CARRYING OUT INVENTION

#### EXAMPLES

The present invention is embodied in more detail by the following examples, but this embodiment is not intended to restrict the present invention. The basic operations and the enzyme reactions with regard to the DNA recombination are carried out according to the literature [Molecular Cloning. A Laboratory Manual", Cold Spring Harbor Laboratory, 1989]. Unless otherwise stated, restrictive enzymes and a variety of modification enzymes to be used were those available from TAKARA SHUZO. The manufacturer's instructions were used for the buffer compositions as well as for the reaction conditions, in each of the enzyme reactions. The cDNA synthesis was carried out according to the literature [Kato, S. et al., Gene 150: 243-250 (1994)].

#### Preparation of Poly(A)<sup>+</sup> RNA

After 1 g of human osteosarcoma cell line Saos-2 cells (ATCC HTB85) was homogenized in 20 ml of a 5.5 M guanidinium thiocyanate solution, 5 mg of mRNA was prepared according to the literature [Okayama, H. et al., "Methods in Enzymology" Vol. 164. Academic Press, 1987]. This was subjected to oligo(dT)-cellulose column chromatography washed with a 20 mM Tris-hydrochloric acid buffer solution (pH 7.6), 0.5 M NaCl, and 1 mM EDTA to obtain 255 µg of a poly(A)<sup>+</sup> RNA according to the literature mentioned above.

#### Preparation of cDNA Library

Ten micrograms of the above described poly(A)<sup>+</sup> RNA were dissolved in a 100 mM Tris-hydrochloric acid buffer solution (pH 8), one unit of an RNase-free bacterial alkaline phosphatase was added, and the reaction was run at 37°C for one hour. After the reaction mixture was subjected to phenol

extraction followed by ethanol precipitation, the pellet was dissolved in a solution containing 50 mM sodium acetate (pH 6), 1 mM EDTA, 0.1% 2-mercaptoethanol, and 0.01% Triton X-100. Thereto was added one unit of a tobacco acid pyrophosphatase (Epicentre Technologies) and a total 100  $\mu$ l volume of the resulting mixture was reacted at 37°C for one hour. After the reaction mixture was subjected to phenol extraction followed by ethanol precipitation, the pellet was dissolved in water to obtain a solution of a decapped poly(A)<sup>+</sup> RNA.

The poly(A)<sup>+</sup> RNA and 3 nmol of a chimeric DNA-RNA oligonucleotide (5'-dG-dG-dG-dG-dA-dA-dT-dT-dC-dG-dA-G-G-A-3') were dissolved in a solution containing 50 mM Tris-hydrochloric acid buffer (pH 7.5), 0.5 mM ATP, 5 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol, and 25% polyethylene glycol, whereto was added 50 units of T4RNA ligase and a total 30  $\mu$ l volume of the resulting mixture was reacted at 20°C for 12 hours. After the reaction mixture was subjected to phenol extraction followed by ethanol precipitation, the pellet was dissolved in water to obtain a chimeric-oligo-capped poly(A)<sup>+</sup> RNA.

After digestion of a vector pKA1 (Japanese Patent Kokai Publication No. 1992-117292) developed by the present inventors with KpnI, about 60 dT tails were added using a terminal transferase. A vector primer to be used below was prepared by digestion of this addition product with EcoRV to remove a dT tail at one side.

After 6  $\mu$ g of the previously-prepared chimeric-oligo-capped poly(A)<sup>+</sup> RNA was annealed with 1.2  $\mu$ g of the vector primer, the resulting mixture was dissolved in a

solution containing 50 mM Tris-hydrochloric acid buffer (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, and 1.25 mM dNTP (dATP + dCTP + dGTP + dTTP), 200 units of a transcriptase (GIBCO-BRL) were added, and the reaction in a total 20 µl volume was run at 42°C for one hour. After the reaction mixture was subjected to phenol extraction followed by ethanol precipitation, the pellet was dissolved in a solution containing 50 mM Tris-hydrochloric acid buffer (pH 7.5), 100 mM NaCl, 10 mM MgCl<sub>2</sub>, and 1 mM dithiothreitol. Thereto were added 100 units of EcoRI and a total 20 µl volume of the resulting mixture was reacted at 37°C for one hour. After the reaction mixture was subjected to phenol extraction followed by ethanol precipitation, the pellet was dissolved in a solution containing 20 mM Tris-hydrochloric acid buffer solution (pH 7.5), 100 mM KCl, 4 mM MgCl<sub>2</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, and 50 µg/ml of the bovine serum albumin. Thereto were added 60 units of an Escherichia coli DNA ligase and the resulting mixture was reacted at 16°C for 16 hours. To the reaction mixture were added 2 µl of 2 mM dNTP, 4 units of an Escherichia coli DNA polymerase I, and 0.1 unit of an Escherichia coli DNase H and the resulting mixture was reacted at 12°C for one hour and then at 22°C for one hour.

Next, the cDNA-synthesis reaction solution was used for transformation of an Escherichia coli DH12S (GIBCO-BRL). The transformation was carried out by an electroporation method. A portion of the transformant was sprayed on the 2xYT agar culture medium containing 100 µg/ml ampicillin and the mixture was incubated at 37°C overnight. A colony formed on the agar medium was picked up at random and inoculated on 2

ml of the 2xYT culture medium containing 100 µg/ml ampicillin. After incubation at 37°C for 2 hours, the mixture was infected with a helper phage MK13K07 (Pharmacia) and incubated further at 37°C overnight. The culture solution was centrifuged to separate the mycelia and the supernatant, wherein a double-stranded DNA was isolated from the mycelia by the alkaline hydrolysis method and a single-stranded plasmid DNA from the supernatant according to the conventional method. After double digestion with EcoRI and NotI, the double-stranded plasmid DNA was subjected to 0.8% agarose gel electrophoresis to determine the size of the cDNA insert. On the other hand, after the sequence reaction using an M13 universal primer labeled with a fluorescent dye and a Taq polymerase (a kit of Applied Biosystems), the single-stranded phage DNA was examined with a fluorescent DNA sequencer (Applied Biosystems) to determine the about 400 bp base sequence at the 5'-terminus of the cDNA. The sequence data were filed as the Homo-Protein cDNA Bank database.

#### cDNA Cloning

The base sequencing of the clones selected at random from the above-mentioned cDNA library was carried out and the obtained base sequence was converted to three frames of the amino acid sequence, which were subjected to a search of the protein data base. The analysis software used was GENETYX-MAC (Software Development). As the result, a protein encoded by a plasmid PHP00966 contained in the clone HP00966 was revealed to be highly homologous to the membrane antigen TM4 superfamily. The structure of this plasmid is depicted in Figure 1. The existence of a 762-bp open reading frame

(Sequence No. 2) was found from the determination of the whole base sequence of the cDNA insert. The open reading frame codes for a protein consisting of 253 amino acid residues and the search of the protein data base using this sequence revealed a 32.5% homology to the human CD63 antigen amino acid sequence over the whole regions. Table 1 shows the comparison between the amino acid sequence of the human membrane antigen TM4 superfamily protein of the present invention (TM4) and that of the human CD63 antigen (CD63). Therein, the marks of -, \*, and . represent a gap, an amino acid residue identical with the protein of the present invention, and an amino acid residue similar to the protein of the present invention, respectively. Furthermore, Figure 2 depicts the hydrophobicity profile of the present protein obtained by the Kyte & Doolittle method. Highly hydrophobic regions of the putative transmembrane domains can be seen at four sites. The characteristics of this pattern are common to other TM4 proteins.



Table 1

```

TM4  MGQCGITSSKTVLVFLNLI FWGAAGILCYVGAYVFITYDDYDHFEDVYTLIPAVVIIAV
      * . * * * * * * * * . * . * * * * *
CD63  AVEGGMKCVKFLLYVLLLAFCAVGLIAGVGGAQLVLSQTIIQGATPGSLLP-VVIIAV
TM4   GALLFIIGLIGCCATIRESRCGLATFVIILLLVFVTEVVVVVLGYVYRAKVENEVDRSIQ
      * . * * . . . * * . * * * * * * * * * * * * * * * * * *
CD63  GVFLFLVAFVGGCGACKENYCLMITFAIFLSLIMLVEVAAAIAGYVFRDKVMSEFNNNFR
TM4   KVKTYNGTNPDAASRAIDYVQRQLHCCGIHNYSDWENTDWFKETKNQSVPLSCCRETAS
      . . . * . . . . . * . * . . * * * * * * * * * * * * * *
CD63  QQMENY--PKNNHTASILD RMQADF KCCGAANYTDWEK---IPSMKNRVPDSCCINVTV
TM4   NCNGSLAHPSDLYAGGCBALVVKKLQEI MMHVIWAALAFAAIQLLGMLCACIVLCRRSRD
      . * . . . . . * * * . . * . . . * * * * * * * * * * *
CD63  GCGINF-NEKAIHKEGCVKIGGWLRKNVLVAAAAALGIAFVEVLGIVFAC-CLVKSIR-
TM4   PAYELLITGGTYA
      . . * * .
CD63  SGYBVM

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### Protein synthesis by In Vitro Translation

The vector pHP00966 having the cDNA of the present invention was used for in vitro translation with a T<sub>m</sub>T rabbit reticulocyte lysate kit (Promega). In this case, [<sup>35</sup>S]methionine was added to label the expression product with a radioisotope. Each of the reactions was carried out according to the protocols attached to the kit. Two micrograms of the plasmid pHP00966 was reacted at 30°C for 90 minutes in a total 100 µl volume of the reaction mixture containing 50 µl of the T<sub>m</sub>T rabbit reticulocyte lysate, 4 µl of a buffer solution (attached to the kit), 2 µl of an amino

acid mixture (Met-free), 8  $\mu$ l of [ $^{35}$ S]methionine (Amersham) (0.37 Mbq/ $\mu$ l), 2  $\mu$ l of T7RNA polymerase, and 80 U of RNasin. To 3  $\mu$ l of the resulting reaction mixture was added 2  $\mu$ l of the SDS sampling buffer (125 mM Tris-hydrochloric acid buffer, pH 6.8, 120 mM 2-mercaptoethanol, 2% SDS solution, 0.025% bromophenol blue, and 20% glycerol) and the resulting mixture was heated at 95°C for 3 minutes and then subjected to SDS-polyacrylamide gel electrophoresis. Determination of the molecular weight of the translation product by carrying out the autoradiography indicated that the cDNA of the present invention yielded the translation product with the molecular mass of about 29 kDa. This value is consistent with the molecular weight of 28,017 predicted for the putative protein from the base sequence represented by Sequence No. 1, thereby indicating that the cDNA certainly codes for the protein represented by Sequence No. 1.

#### INDUSTRIAL APPLICATION

The present invention provides a human membrane antigen TM4 superfamily protein and a cDNA encoding said protein. The protein of the present invention can be used as a pharmaceutical for treatment and diagnosis of cancers and also as an antigen for preparation of an antibody against said protein. Further, said DNA can be used for the expression of a large amount of said protein.

## SEQUENCE LISTING

Sequence No.: 1

Sequence length: 759

Sequence type: Nucleic acid

Strandedness: Double

Topology: Linear

Sequence kind: cDNA to mRNA

Sequence description

ATG GGC CAG TGC GGC ATC ACC TCC TCC AAG ACC GTG CTG GTC TTT CTC	48
Met Gly Gln Cys Gly Ile Thr Ser Ser Lys Thr Val Leu Val Phe Leu	
1 5 10 15	
AAC CTC ATC TTC TGG GGG GCA GCT GGC ATT TTA TGC TAT GTG GGA GCC	96
Asn Leu Ile Phe Trp Gly Ala Ala Gly Ile Leu Cys Tyr Val Gly Ala	
20 25 30	
TAT GTC TTC ATC ACT TAT GAT GAC TAT GAC CAC TTC TTT GAA GAT GTG	144
Tyr Val Phe Ile Thr Tyr Asp Asp Tyr Asp His Phe Phe Glu Asp Val	
35 40 45	
TAC ACG CTC ATC CCT GCT GTA GTG ATC ATA GCT GTA GGA GCC CTG CTT	192
Tyr Thr Leu Ile Pro Ala Val Val Ile Ile Ala Val Gly Ala Leu Leu	
50 55 60	
TTC ATC ATT GGG CTA ATT GGC TGC TGT GCC ACA ATC CGG GAA AGT CGC	240
Phe Ile Ile Gly Leu Ile Gly Cys Cys Ala Thr Ile Arg Glu Ser Arg	
65 70 75 80	
TGT GGA CTT GCC ACG TTT GTC ATC ATC CTG CTC TTG GTT TTT GTC ACA	288
Cys Gly Leu Ala Thr Phe Val Ile Ile Leu Leu Val Phe Val Thr	
85 90 95	

GAA GTT GTT GTA GTG GTT TTG GGA TAT GTT TAC AGA GCA AAG GTG GAA	336
Glu Val Val Val Val Val Leu Gly Tyr Val Tyr Arg Ala Lys Val Glu	
100 105 110	
AAT GAG GTT GAT CGC AGC ATT CAG AAA GTG TAT AAG ACC TAC AAT GGA	384
Asn Glu Val Asp Arg Ser Ile Gln Lys Val Tyr Lys Thr Tyr Asn Gly	
115 120 125	
ACC AAC CCT GAT GCT GCT AGC CGG GCT ATT GAT TAT GTA CAG AGA CAG	432
Thr Asn Pro Asp Ala Ala Ser Arg Ala Ile Asp Tyr Val Gln Arg Gln	
130 135 140	
CTG CAT TGT TGT GGA ATT CAC AAC TAC TCA GAC TGG GAA AAT ACA GAT	480
Leu His Cys Cys Gly Ile His Asn Tyr Ser Asp Trp Glu Asn Thr Asp	
145 150 155 160	
TGG TTC AAA GAA ACC AAA AAC CAG AGT GTC CCT CTT AGC TGC TGC AGA	528
Trp Phe Lys Glu Thr Lys Asn Gln Ser Val Pro Leu Ser Cys Cys Arg	
165 170 175	
GAG ACT GCC AGC AAT TGT AAT GGC AGC CTG GCC CAC CCT TCC GAC CTC	576
Glu Thr Ala Ser Asn Cys Asn Gly Ser Leu Ala His Pro Ser Asp Leu	
180 185 190	
TAT GCT GAG GGG TGT GAG GCT CTA GTA GTG AAG AAG CTA CAA GAA ATC	624
Tyr Ala Glu Gly Cys Glu Ala Leu Val Val Lys Lys Leu Gln Glu Ile	
195 200 205	
ATG ATG CAT GTG ATC TGG GCC GCA CTG GCA TTT GCA GCT ATT CAG CTG	672
Met Met His Val Ile Trp Ala Ala Leu Ala Phe Ala Ala Ile Gln Leu	
210 215 220	
CTG GGC ATG CTG TGT GCT TGC ATC GTG TTG TGC AGA AGG AGT AGA GAT	720
Leu Gly Met Leu Cys Ala Cys Ile Val Leu Cys Arg Arg Ser Arg Asp	
225 230 235 240	

CCT GCT TAC GAG CTC CTC ATC TACT GGC GGA ACC TAT GCA  
Pro Ala Tyr Glu Leu Leu Ile Thr Gly Gly Thr Tyr Ala

759

245

250

Sequence No.: 2

Sequence length: 1722

Sequence type: Nucleic acid

Strandedness: Double

Topology: Linear

Sequence kind: cDNA to mRNA

Origin:

Animal name: Homo sapiens

Cell kind: Osteosarcoma

Cell line: Saos-2

Clone name: HP00966

Sequence characteristics:

Characterization code: CDS

Existence position: 156..917

Characterization method: E

Sequence description

ACTTGCTGGG GTCGGGGCTG CGCGACGGCG CAGGGGCTGC GGGGAGCGCC GCGCAGGCCG 60

TGCAGTTCCT AGCGAGGAGG CGCCGCCGCC ATTGCCGCTC TCTCGGTGAG CGCAGCCCCG 120

CTCTCCGGGC CGGGCCTTCG CGGGCCACCG GCGCC ATG GGC CAG TGC GGC ATC 173

Met Gly Gln Cys Gly Ile

1

5

ACC TCC TCC AAG ACC GTG CTG GTC TTT CTC AAC CTC ATC TTC TGG GGG 221

Thr Ser Ser Lys Thr Val Leu Val Phe Leu Asn Leu Ile Phe Trp Gly

10

15

20

GCA GCT GGC ATT TTA TGC TAT GTG GGA GCC TAT GTC TTC ATC ACT TAT	269
Ala Ala Gly Ile Leu Cys Tyr Val Gly Ala Tyr Val Phe Ile Thr Tyr	
25 30 35	
GAT GAC TAT GAC CAC TTC TTT GAA GAT GTG TAC ACG CTC ATC CCT GCT	317
Asp Asp Tyr Asp His Phe Phe Glu Asp Val Tyr Thr Leu Ile Pro Ala	
40 45 50	
GTA GTG ATC ATA GCT GTA GGA GCC CTG CTT TTC ATC ATT GGG CTA ATT	365
Val Val Ile Ile Ala Val Gly Ala Leu Leu Phe Ile Ile Gly Leu Ile	
55 60 65 70	
GGC TGC TGT GCC ACA ATC CGG GAA AGT CGC TGT GGA CTT GCC ACG TTT	413
Gly Cys Cys Ala Thr Ile Arg Glu Ser Arg Cys Gly Leu Ala Thr Phe	
75 80 85	
GTC ATC ATC CTG CTC TTG GTT TTT GTC ACA GAA GTT GTT GTA GTG GTT	461
Val Ile Ile Leu Leu Leu Val Phe Val Thr Glu Val Val Val Val Val	
90 95 100	
TTG GGA TAT GTT TAC AGA GCA AAG GTG GAA AAT GAG GTT GAT CGC AGC	509
Leu Gly Tyr Val Tyr Arg Ala Lys Val Glu Asn Glu Val Asp Arg Ser	
105 110 115	
ATT CAG AAA GTG TAT AAG ACC TAC AAT GGA ACC AAC CCT GAT GCT GCT	557
Ile Gln Lys Val Tyr Lys Thr Tyr Asn Gly Thr Asn Pro Asp Ala Ala	
120 125 130	
AGC CGG GCT ATT GAT TAT GTA CAG AGA CAG CTG CAT TGT TGT GGA ATT	605
Ser Arg Ala Ile Asp Tyr Val Gln Arg Gln Leu His Cys Cys Gly Ile	
135 140 145 150	
CAC AAC TAC TCA GAC TGG GAA AAT ACA GAT TGG TTC AAA GAA ACC AAA	653
His Asn Tyr Ser Asp Trp Glu Asn Thr Asp Trp Phe Lys Glu Thr Lys	
155 160 165	

AAC CAG AGT GTC CCT CTT AGC TGC TGC AGA GAG ACT GCC AGC AAT TGT 701  
 Asn Gln Ser Val Pro Leu Ser Cys Cys Arg Glu Thr Ala Ser Asn Cys  
 170 175 180  
 AAT GGC AGC CTG GCC CAC CCT TCC GAC CTC TAT GCT GAG GGG TGT GAG 749  
 Asn Gly Ser Leu Ala His Pro Ser Asp Leu Tyr Ala Glu Gly Cys Glu  
 185 190 195  
 GCT CTA GTA GTG AAG AAG CTA CAA GAA ATC ATG ATG CAT GTG ATC TGG 797  
 Ala Leu Val Val Lys Lys Leu Gln Glu Ile Met Met His Val Ile Trp  
 200 205 210  
 GCC GCA CTG GCA TTT GCA GCT ATT CAG CTG CTG GGC ATG CTG TGT GCT 845  
 Ala Ala Leu Ala Phe Ala Ala Ile Gln Leu Leu Gly Met Leu Cys Ala  
 215 220 225 230  
 TGC ATC GTG TTG TGC AGA AGG AGT AGA GAT CCT GCT TAC GAG CTC CTC 893  
 Cys Ile Val Leu Cys Arg Arg Ser Arg Asp Pro Ala Tyr Glu Leu Leu  
 235 240 245  
 ATC ACT GGC GGA ACC TAT GCA TAGTTGACAA CTCAAGCCTG AGCTTT 940  
 Ile Thr Gly Gly Thr Tyr Ala  
 250  
 TTGGTCTTGT TCTGATTTGG AAGGTGAATT GAGCAGGTCT GCTGCTGTTG GCCTCTGGAG 1000  
 TTCATTTAGT TAAAGCACAT GTACACTGGT GTTGGACAGA GCAGCTTGGC TTTTCATGTG 1060  
 CCCACCTACT TACCTACTAC CTGCGACTTT CTTTTTCCTT GTTCTAGCTG ACTCTTCATG 1120  
 CCCCTAAGAT TTTAAGTACG ATGGTGAACG TTCTAATTTT AGAACCAATT GCGAGTCATG 1180  
 TAGTGTGGTA GAATTAAAGG AGGACACGAG CCTGCTTCTG TTACCTCCAA GTGGTAACAG 1240  
 GACTGATGCC GAAATGTCAC CAGGTCTTTT CAGTCTTCAC AGTGGAGAAC TCTTGGCCAA 1300  
 AGGTTTTTGC GGGGAGGAGG AGGAAACCAG CTTTCTGGTT AAGGTTAACA CCAGATGGTG 1360  
 CCCCTCATTG GTGTCCTTTT AAAAAATATT TACTGTAGTC CAATAAGATA GCAGCTGTAC 1420  
 AAAATGACTA AAATAGATTG TAGGATCATA TGGGCTATAT GTTGGTTGAT GTTCAAAATG 1480

AGAGACTGAG CTTTGAAACT AGTGGTTTTT AATCAAAGTT GGCTTTATAG GAGGAGTATA 1540  
ATGTATGCAC TACTGTTTTA AAAGAATTAG TGTGAGTGTG TTTTGTATG AATGAGCCCA 1600  
TTCATGGTAA GTCTTAAGCT TGTGGAAT AATGTACCCA TGTAGACTAG CAAAATAGTA 1660  
TGTAGATGTG ATCTCAGTTG TAAATAGAAA AATCTAATTC AATAAACTCT GTATCAGCCC 1720  
CC 1722



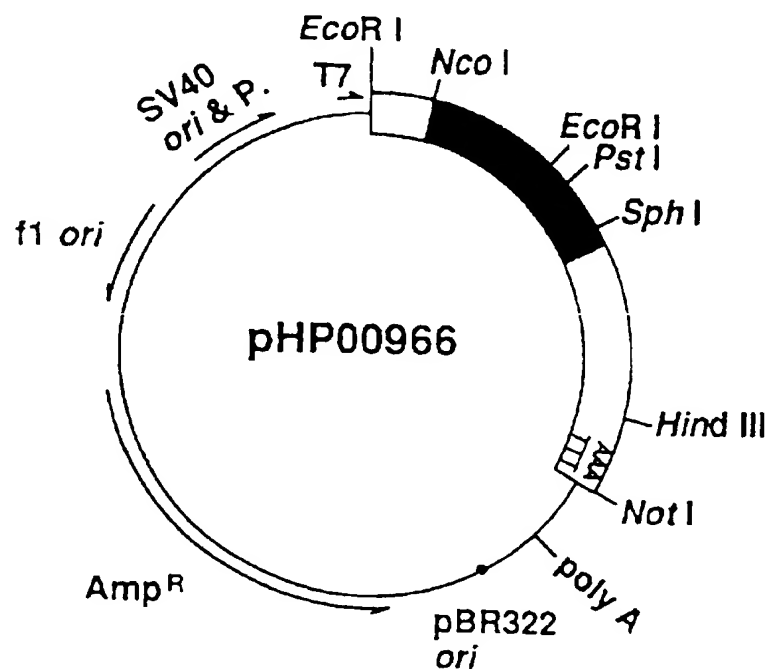
## CLAIMS

1. A protein comprising an amino acid sequence represented by Sequence No. 1, optionally with deletion, substitution and/or addition of at least one amino acid residue therein.

2. A DNA coding for the protein according to Claim 1, or a DNA hybridizable therewith.

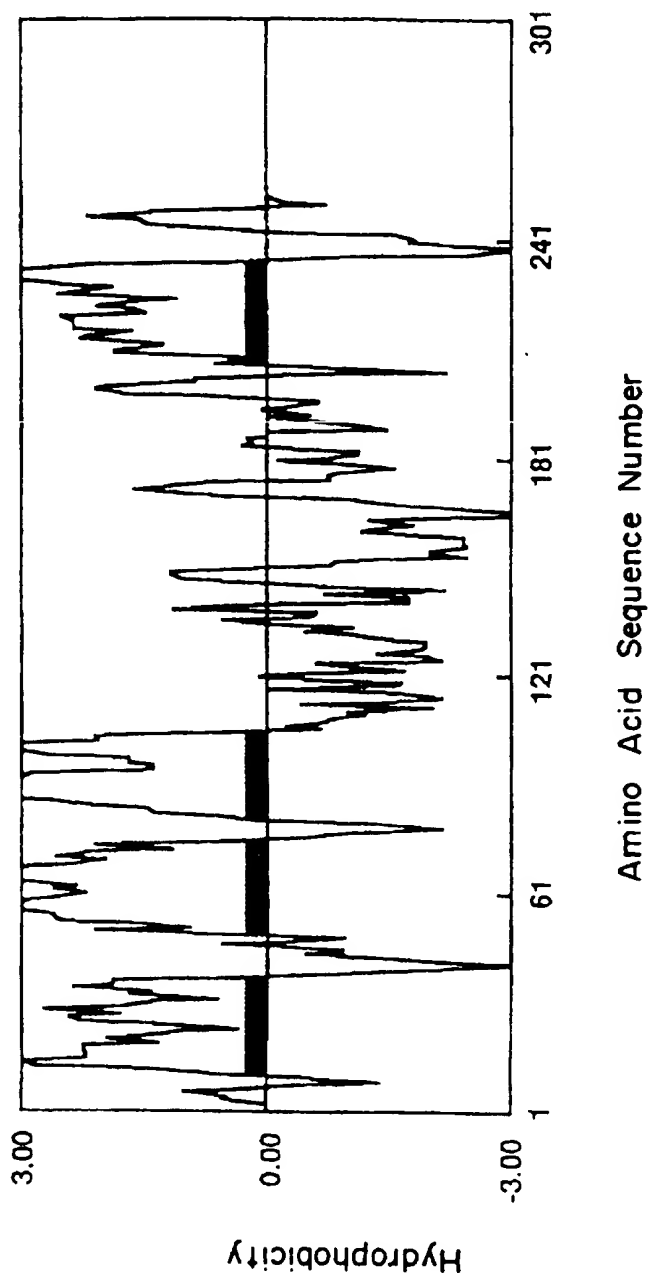
3. A cDNA comprising a base sequence represented by Sequence No. 1, or a cDNA hybridizable therewith.

4. A cDNA according to Claim 3 which comprises a base sequence represented by Sequence No. 2, or a cDNA hybridizable therewith.

*Fig. 1*

2 / 2

Fig. 2



# INTERNATIONAL SEARCH REPORT

International Application No.  
JP 97/00811

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 6 C12N15/12 C07K14/705 //A61K38/17,G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 26, 1991, pages 17566-17572, XP002037858 WU C. AND NOGUCHI C. T.: "Activation of globin gene expression by cDNAs from induced K562 cells." see figure 8 & EMBL Database entry Hsggefera Accession number M69023; 1993 WU C. AND NOGUCHI C.T.	1-4
A	--- JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 5, 1991, pages 3239-3245, XP002037856 METZELAAR M.J. ET AL.: "CD63 antigen." cited in the application see the whole document, especially Fig. 3 --- -/--	1-4

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

\* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
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- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

19 August 1997

Date of mailing of the international search report

29.08.97

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Name and address of the applicant

Mandil, B

# INTERNATIONAL SEARCH REPORT

International Application No.

97/00811

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>EMBL Database entry Mmaa98284;                      ;Accession number AA098284;                      27.Okt.1996; Marra M. et al.: 'The WashU-                      HHMI Mouse EST project.'                      XP002037859                      see abstract</p> <p style="text-align: center;">---</p>	1-4
T	<p>FASEB JOURNAL,                      vol. 11, no. 6, May 1997,                      pages 428-442, XP002037857                      MAECKER H.T. ET AL. : "The tetraspanin                      superfamily: molecular facilitators."                      see the whole document</p> <p style="text-align: center;">-----</p>	1-4